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ESTIMATING THE BIODEGRADATION KINETICS BY MIXED CULTURE DEGRADING PYRENE (Pyr)

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Abstract
Biodegradation and kinetics of Pyrene (Pyr) degradation by a mixed culture previously isolated from hydrocarbon-polluted soil were conducted. Preliminary investigation on environmental factors affecting the degradation of Pyr such as temperature, pH and concentrations of Pyr was performed. These factors were optimised and established in aqueous experiments. In order to develop kinetics of Pyr degradation, an optimum temperature of 30^{O}C and pH of 7.0 was used. Biodegradation kinetics was carried out, at first, using higher concentration between (100-700 ppm) as sole source of carbon in mineral salt medium (MSM) supplemented with 0.1% yeast extract. The result indicated that a range of concentration between (100-700 ppm) inhibits the performance of the mixed culture. A concentration range between (10-100 ppm) did not inhibit the growth of the mixed culture. A First-order rate constant, k was higher (0.0487 mg/lh) with a substrate concentration of 20 ppm than other concentrations. The average degradation rate constant is 0.0029 mg/Lh for all the concentrations tested. This indicated that the mixed culture could degrade over 0.0696 ppm of Pyr per day. It also confirmed that kinetics of microbial degradation was partially fitted into Monod model. The data can be used to estimate biodegradation of Pyr by a mixed culture and preliminarily estimation of degradation rates.

Keywords: Pyrene, Mixed culture, Degradation, Kinetics, Monod Equation

1. Introduction

Microbial growth kinetics is a very useful tool to describe the characteristic of microbial growth and degradation of organic compounds; they also assist in determining the extent of remediation, as in the case of field bioremediation. Kinetics models usually describe the amount of chemical compounds degraded by pure cultures of microorganism or in particular, bacteria. Many attempts have been made to model and describe bacterial biodegradation of organic compounds. For examples, models such as first-order model, second-order model; in the case of second-model, it is used when the rate of substrate concentration is much more lower than affinity constant (K_s), and the relationship is described by the second-order model, and is given by equation 1.

$$-\frac{d[S]}{dt} = \frac{\mu_{max}}{YK_S}[B][S] \cong k[B][S] \quad \text{.....1}$$

where k is a second-order rate constant (mg/Lh) (Paris *et al*. 1981). Also, an integrated Michaelis-Menten (Michaelis and Menten 1913) was applied to estimate enzymatic kinetics parameters based on enzyme acting on a single substrate with neither reverse nor product inhibition (Liao *et al*. 2005). This combination is given by

equation 2.

$$\left(\frac{\mathrm{In}\left(\frac{S_o}{S}\right)+(S_o-S)}{K_m}\right)=\left(\frac{V_m}{K_m}\right)t \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

Robinson and Tiedje, (1983) used integrated Monod and logistic models to estimate degradation kinetics.

In the fields of bioremediation, Monod kinetics (Monod 1949) that was originally derived from a pure cultures and single substrates, are usually used to describe the behaviour of undefined or defined mixed cultures growing with single substrate or complex substrate mixtures (Kovarova-Kovar and Egli 1998). But, Monod equation is purely empirical and is proposed based on curve fitting, therefore has poor theoretical explanation of the physical meaning of the Monod constant; there is a large variation observed on this constant (Liu, 2007). Besides, the equation only relates the growth rate to the concentration of a single growth-controlling substrate through two parameters, the maximum specific growth rate (μ_{max}), and the substrate affinity constant (K_S). After all, growth is a result of catabolic and anabolic enzymatic activities and can be quantitatively represented on the bases of growth models (Kovarova-Kovar and Egli 1998). Although there are criticisms surrounding the use of Monod equation, however, Bailey and Ollis as quoted by Liu, (2007) observed "it is apparent that the Monod equation is probably a great oversimplification. As in other areas of engineering, however, this is a case where a relatively simple equation reasonably expresses an interrelationship, even though the physical meaning of the model parameters is unknown or perhaps does not exist". Kovarova-Kovar and Egli, (1998) observed "classical Monod equation does not see perhaps cells need substrate or synthesize product even while they do not grow". That was why many modifications were introduced to the equation, such as maintenance and threshold concentration.

Considering the Monod equation mathematically, it is quite analogous to the formula proposed by Michelis and Menten to describe enzyme kinetics, but the meaning of K_s and K_m is quite different. While Monod had already highlighted that there is no relationship between the K_s (affinity constant used in his growth model that represents the substrate concentration at $\mu = 0.5\mu_{max}$) and the Michaelis-Menten constant K_m. Whereas Michealis-Menten constant K_m describes a process catalysed by a single enzyme, Monod kinetics describe growth process that links growth and growth-linked biodegradation of more complex system in nature involving many enzymes (Kovarova-Kovar and Egli 1998). Consequently, frequent description of kinetics of growth or growth-related biodegradation as "Michaelis-Menten-type", is kinetically not correct. Exception to this, is in a special case when cell growth is controlled by the rate of active transport of a substrate, perhaps K_s can be considered to be similar to the Michealis-Menten constant (K_m) and $1/K_s$, and is interpreted as a reflection of the affinity of the cell towards a substrate (Kovarova-Kovar and Egli 1998).

Applications of Monod-like model for the kinetics of PAHs degradation were reported in many works. For instance, Gomes *et al*. (2006) tested kinetics of fluorene degradation using fitting of zero order model, first-order model and saturation model; Volkering *et al*. (1992) used 4th order Runge-Kutta method to compare measured values. Chen

et al. (2008) used multi-factors on degradation kinetics of (PAHs) by *Sphingomonas* sp and reported degradation of phenanthrene best described by first-order rate model with a K value of 0.1185. In the case of mixed cultures and mixtures of substrates, the degradation of the compounds by the mixed culture is term "black box system" (Dimitriou-Christidis 2005). Although in pure cultures, the experimental constants were variously reported; notwithstanding, the constants could still represent growth conditions. Nonetheless, (Kovarova-Kovar and Egli 1998), questioned the integrity of applying kinetics constants that were traditionally based on growth control by a single substrate to real environmental situation where cells are competing for varieties of carbon sources. However, where the medium consists of mixed culture and mixed substrate, it is difficult and sometimes misleading to describe the process kinetic by a single set of kinetic constant (Kovarova-Kovar and Egli 1998). Obviously, particular organism can successfully compete in the environment if it can change and adapt adequately its kinetics properties.

In the case of mixed culture degrading a single or mixture of polycyclic aromatic hydrocarbon (PAH) has only been attempted recently (Reardon *et al*. 2002). The authors reported that mixed culture degrade mixtures of PAH faster than pure culture alone, but poorly degraded a single compound, toluene or phenol. Study conducted by Lotfabad and Gray (2002) reported that a competitive model was able to described kinetics of PAHs degradation from a creosote-contaminated soil. Although argued that Kinetics of PAHs degradation by mixed culture could follow competitive-inhibition, but failed to explain the underlying mechanism. The objective of this study was to investigate the effect of concentration on the biodegradation of Pyr by a mixed culture and also to describe the kinetics of Pyr degradation.

2. Materials and Method

2.1 Materials

Chemicals for the preparation of mineral salt medium and other chemicals such as Pyr 98% purity, dichloromethane (DCM) high performance liquid chromatography (HPLC) standard and analytical reagent grade methanol, hexane and acetone were purchased from Fisher Scientific (Malaysia). The chemicals were weighed and the appropriate solutions were made according to mineral salt medium reported by (Tao *et al*. 2007). A Pyr stock solution was prepared by dissolving in acetone at a concentration of 10,000 ppm in an amber brown bottle; and stored at 4^{O}C until use. The media for the inoculation development were also prepared using MSM supplemented with 0.1% yeast extract. The pH of the media was adjusted to pH of 7.2.

2.2 Preparation of Inoculum

The mixed culture was taken from the culture stock and thawed; the fresh culture was regrown aseptically with 10 ppm of Pyr in MSM as sole carbon source. The grown culture was harvested as soon as it reaches the late exponential phase with optical density OD (OD_{600nm} 0.8-0.9). Harvesting was done using a centrifuge at 8000 g for 10 min at 4^{O}C, and then the supernatant was aspired carefully. The cells were washed three

times with phosphate buffer and re-suspended in MSM. Finally, 1% v/v of the inoculum was used for the growth kinetics test.

2.3 Determination of Degradation Kinetics

A 250 mL conical flask containing a 100 mL mineral salt medium supplemented with 0.1% yeast extract was sterilised. After cooling, 100 ppm of Pyr was poured aseptically by filtering through 45 µm cellulose acetate filter paper. The sample was inoculated with 1% v/v and incubated in an incubator shaker at 30^{o}C, 180 rpm (agitation) for a week. This is used as an inoculum for the kinetics experiments.
For the kinetics test, a 250 ml conical flasks containing MSM supplemented with 0.1% yeast extracts were aseptically prepared with an initial concentrations of a Pyr concentration of: 10, 20, 40, 60, 80, 100, 200, 300, 400, 500, 600, and 700 ppm respectively. The triple flasks were inoculated with 2% v/v of inoculum incubated at 30^{O}C with 180 rpm and monitored over 20 days. Samples were collected at interval of every alternate day. The growth was monitored spectrophometrically (HACH, DR/2500), and the optical densities were converted to dry weight using a correlation curve of dry weight against optical densities at 600nm.

2.4 Extraction and Analysis of Pyr

A stock solution of 400 ppm of Pyr was prepared by dissolving it in a HPLC grade DCM. Different concentrations were prepared by diluting to 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ppm. Selecting different wavelength ranges between 200-580 nm ran a UV-visible spectrophotometer, the UV-visible spectrophotometer was zeroed using DCM as blank and then each concentration was tested. Consequently, for the determination of residual concentration of Pyr, a correlation curve was obtained by plotting absorbance at 243 nm against concentration. A 2 mL of aqueous sample was extracted with DCM three times in equal volume. Aqueous sample was filtered with pre-dried anhydrous sodium sulphate (dried at 200^{O}C for 2 h) in a funnel lined with glass-wool bed filter. The filtrate was collected and subjected to concentration step using a rotary evaporator at reduced pressure and concentrated to 1 ml under slow nitrogen purging. Finally, the residual Pyr concentration was quantified using the correlation curve.

2.5 Mathematical Approach

Cell growth and substrate consumptions were usually model based on establishing favorable growth conditions for an organism. If the condition is favorable, the growth rate is expressed as the change of population with time and is usually after the ”lag time” or time interval between inoculation and growth at the characteristics rate. So, at the beginning, the number of cells is X_0 and the value of the characteristics time for the population to double in a batch culture is t_d, and the resultant population present in the medium will be

$$X=X_o e^{\mu t} \quad \ldots\ldots 3$$

and the population doubling time t_d is

$t_d = In\frac{2}{\mu}$.....4

where the population may increase at a rate μ without any change in the biomass, may result in a longer time, τ, that would be required for the population to double; and is given as the reciprocal of specific growth rate:

$\tau = \frac{1}{\mu} = 1.45td$.....5

Where τ is referred to as birth time or interval that an organism must survive before dividing in a medium of population that has particular growth rate (Button 1985, Pitter and Chudoba 1990).

When differentiating equation 3, it gives

$\frac{dX}{dt} = \mu X$.....6

where μ is the instantaneous growth rate constant and X is bacterial biomass expressed in wet weight or dry weight per litre (Button 1985, Pitter and Chudoba 1990). Equation 4 is the basic differential equation of microbial growth. The relationship defining the specific growth rate μ can be obtained from equation 2 and is given as

$\mu = \frac{dX}{dt}\frac{1}{X}$.....7

In order to solve for the value of μ, equation 7 is integrated with respect to X and t using initial and final microbial population with time and the result is

$\int_{X_o}^{X}\frac{dX}{X} = \mu\int_{o}^{t} dt$.....8

and by integrating this equation 8, will give rise to a half-logarithmic (base 10) plot of log X versus time:

$InX = \mu t + InX_o$.....9

This equation 9 is a straight-line equation with coordinates $y = InX$ and $X = t$ and the slope is μ. It can then hold that:

$\mu = \frac{InX - InX_o}{t}$.....10

and this equation is used to calculate the specific growth rate (Pitter & Chudoba 1990). But, when an organism grows based on substrate consumption, and in this case Pyr, the growth is assumed to depend on the rate of depletion of the Pyr with time; because, the bacteria grow by assimilation of organic carbon as source of carbon and energy. The reduction in concentration of Pyr can be described by

$\frac{dPyr}{dt} = -Kt$.....11

where Pyr= concentration of Pyr in the culture medium and K is the degradation constant and t is the time taken for the Pyr to be consumed by the mixed culture in the medium. Therefore, all the degradation data obtained were fitted into equation 11 so as to estimate the degradation rate constant, K. The biomass concentration was estimated based on the correlation curve obtained with r^2 0.998 and μ was calculated according equation 9, subsequently μ_{max} was also estimated from the graph obtained by plotting μ against time. The growth data were fitted also into the famous (Monod 1949) equation, which is described by the following equation 12

$= \frac{\mu_{max}S}{K_s + S}$.....12

This equation is derived based on growth rate depending on the limiting concentration of a limiting substrate, S and μ_{max}, which is the maximum specific growth rate per time, and K_S= half-velocity constant, per volume, numerically equal to the substrate concentration at which $\mu = \mu_{max}/2$. So, with this understanding of microbial growth, the kinetics of degradation of Pyr was attempted to produce rate equation that could describe the order of reaction and yield a specific reaction rate or rate constant (k) (Battersby 1990).

3. Results and Discussion

3.1 Pre-screening of suitable Pyr concentration for kinetics study

The result indicated that at higher concentrations, the mixed culture growth was very low and inconsistent. This could be attributed to inhibitory effect of high Pyr concentration on the performance of the mixed culture as can be seen from the biomass growth in Figure, 3.1 (A, B, C, D, F, and G) respectively. A comparison among the biomass growth curves in Figure 3.1 (A, B, C and D) indicated that the growth was very consistent with 100 ppm than between 200 ppm and 400 ppm.

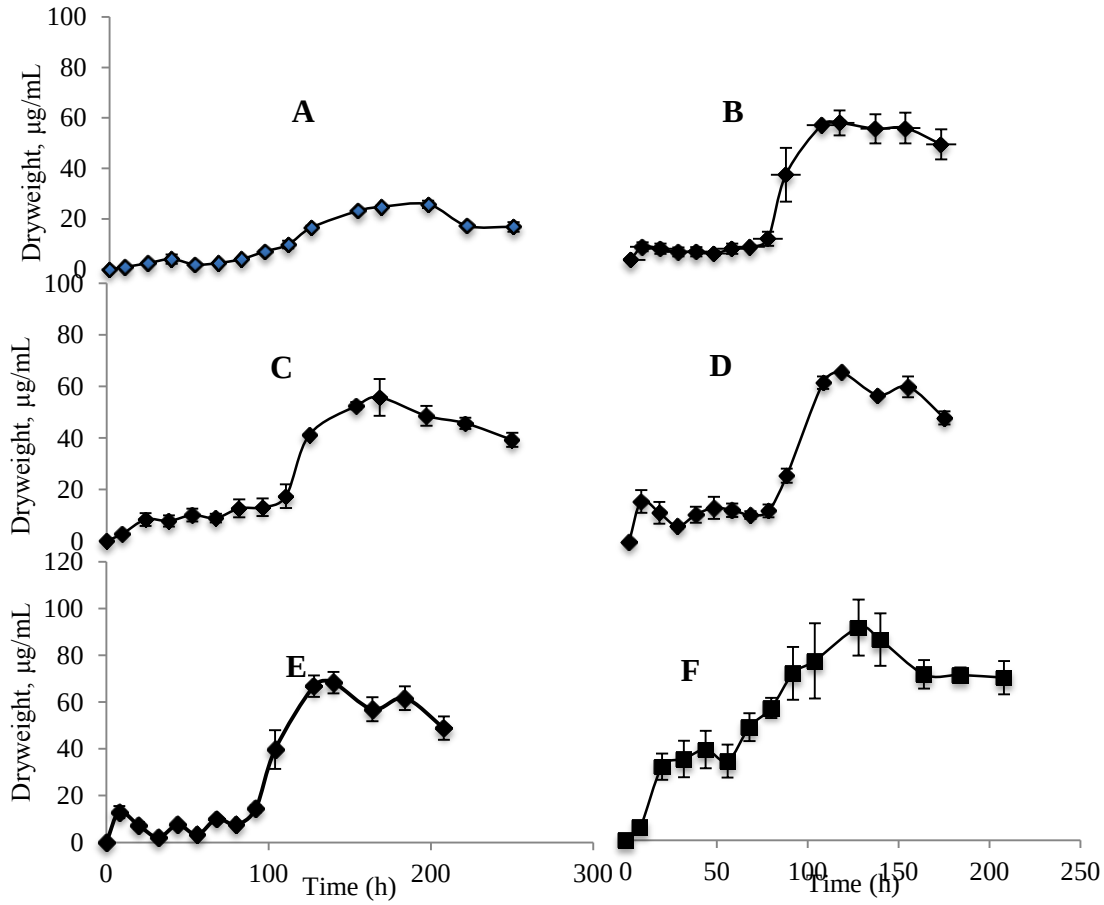

Figure 1: Effect of Concentrations of Pyr ((A) 100 ppm; (B) 200 ppm; (C) 300 ppm: (D) 400 ppm; (E) 500 p m; (F) 600 ppm; and (G) 700 ppm) on average biomass growth with time at 30^{O}C, pH 7.0 and 180 rpm incubated in the dark; Each data point represents the mean standard error of three replicates

Although there was growth in these cultures, but there were fluctuations causing interpretation difficult or the growth do not seem to represent real microbial growth; or

the mixed culture could not endure higher Pyr concentration. Because, there is no continuity of biomass growth due to inconsistent increase in population in those cultures between the ranges of (200-400) respectively.

The trend looks similar when examining Figure 1 (E, F and G); the comparison of biomass growth with the substrate concentrations between 500 ppm and 700 ppm indicated a very inconsistent results. There was more biomass growth at 500 ppm than either 600 ppm or 700 ppm. But, the growth at 500 ppm is also fluctuating and not consistent, which undermines real microbial growth conditions. While in the case of 100 ppm, the culture grows continually with clear lag and exponential phases. This is the reason why the concentration above 100 ppm was not chosen for the kinetics studies. These preliminary experiments to determine a better concentration ranges were repeated severally before choosing a substrate concentration below 100 ppm.

From the analysis of the specific growth rate, μ of the mixed culture, using higher substrate concentrations, as shown in Table 1; μ at 100 ppm was higher than at other substrate concentrations above 100 ppm. Using the goodness of fit, and calculated specific growth rates using log numbers of biomass concentrations against time at different substrate concentrations; at a substrate concentration of 100 ppm, a correlation coefficient of above 80% was obtained. On the other hand, calculated values of r^2 for other substrate concentrations were below 33% with a very low and inconsistent μ_{max}. Because, at higher substrate concentrations, the estimated μ_{max} should have been increasing, so that probable inhibition by the effect of substrate concentration wouldn't have been assumed. Subsequently, a choice to use a concentration below 100 ppm was further strengthened by these results. Therefore, another set of experiments was conducted using substrate concentrations below 100 ppm. Consequently, a substrate concentrations range between 10 ppm and 100 ppm was adopted to develop the kinetics.

Table 1: Variation of specific growth rates μ at higher concentration of Pyr

No	Pyr Concentration, ppm	μ $\mu g(Lhr)^{-1}$	r^2	μ_{max} $(\mu g(Lhr)^{-1})$
1	100	0.2950	0.8743	2.5300
2	200	0.06624	0.3385	1.0915
3	300	0.0792	0.2886	1.6831
4	400	0.0558	0.2755	0.8211
5	500	0.1028	0.0584	1.0889
6	600	0.0640	0.2982	0.6127
7	700	0.0911	0.1358	0.5603

From Figure 2 (A and B), comparing biomass growth of 10 ppm and 20 ppm indicated that the growth was higher at 20 ppm than 10 ppm. Even though both concentrations having long lag phase of 96 hr; there was higher maximum growth rate in 20 ppm with a defined log phase. In contrast, there were very minimal biomass growth differences between 40 ppm and 60 ppm despite the effect of

concentration on the biomass growth. This situation was clearly shown in Figure 2 (C and D); where microbial growth for both concentrations (40 and 60 ppm) reached their log phases on the same day. Although, the differences were very small, the biomass growth shows increasing trend: as the concentration increases, the biomass growth also increases. In addition, Figure 2 (E and F) shows another growth pattern; where with a concentration of 100 ppm, the biomass growth showed a shorter lag phase and longer exponential phase. In contrast, at a concentration of 80 ppm, the biomass growth has a longer lag phase and shorter exponential phase. Nonetheless, there was an effect of concentration on the growth pattern of the mixed culture as has been explained earlier.

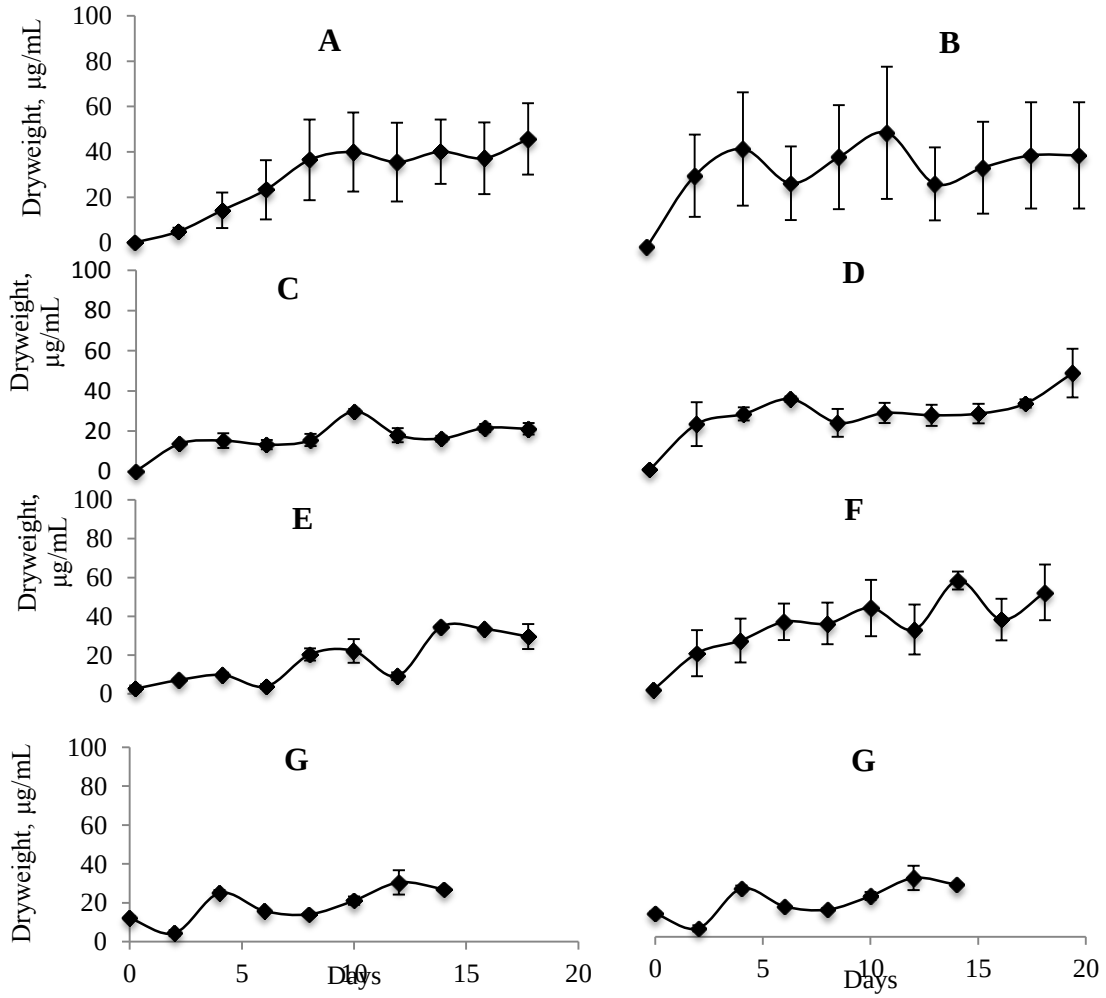

Figure 2: Effect of Concentrations of Pyr at (A) 10 ppm; (B) 20 ppm; (C) 40 ppm;(D) 60 ppm; (E) 80 ppm; (F) 100 ppm on average biomass growth with time at 30^{O}C, pH7.0 and 180 rpm incubated in the dark; Each data point represents the mean standard error of three replicates

3.2 Determination of growth kinetics

In order to determine the kinetics of Pyr degradation by the mixed culture, an inoculum prepared using Pyr supplemented with MSM plus 0.1% yeast extract was inoculated into MSM with 0.1% yeast extract at pH 7.0 containing Pyr with initial

concentrations of (10 ppm -100 ppm) and incubated at 30^{O}C 180 rpm. Samples were taken every 24 h and determined for the residual concentration after extraction and concentration. During the selection of an effective wavelength, a wavelength of 243 nm gave the highest optical densities at different concentrations. The residual Pyr was quantified using the correlation curve developed with r^2, 0.998 as shown in Figure 3. The degradation of Pyr by the mixed culture was monitored with time within the range of concentration between (10 ppm-100 ppm).

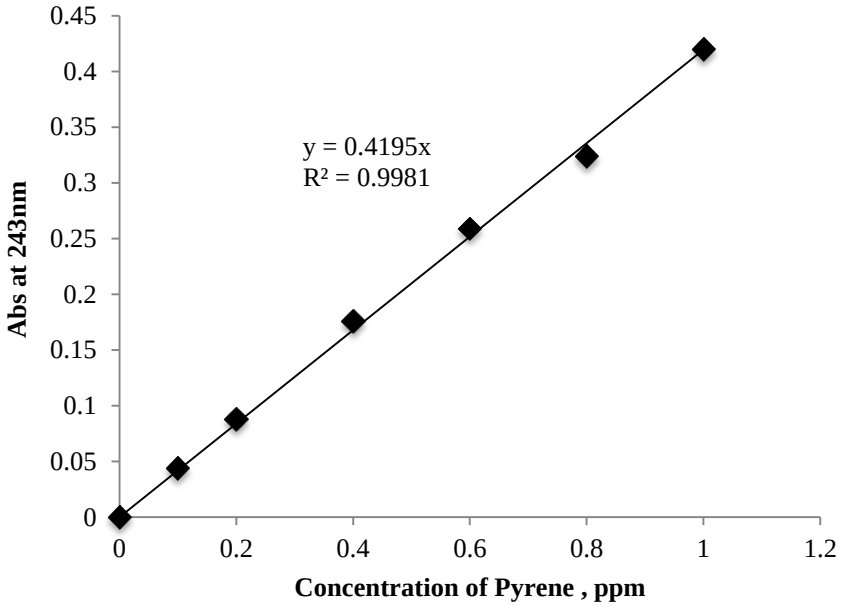

Figure 3: Calibration Curves for Pyr (dissolved in a DCM)

The result of degradation using 10 ppm as shown in Figure 4 (A); at the initial stage, there was slow increase in population due to a very limited period of acclimatisation. At 68 h, Pyr dropped by about 40%, but at the end of 100 h, over 50% were degraded. From 140 h-168 h, the declining in Pyr concentration was stabilised to 60% and the population of the mixed culture reached their stationary phase (30µg/mL). From Figure 4 (B), the degradation did not start until 96 h, this indicates that at 20 ppm, there was a clear lag phase or the mixed culture acclimatised very slow. Beyond 100 h, Pyr concentration dropped to nearly 50% and continued to reduce to below 20%. The population growth was also higher compared to the growth at 10 ppm. In contrast, when the initial concentration was 40 ppm, as shown in Figure 4 (C), there was no clear lag phase and the degradation of Pyr started early from 24 h. It continued to reduce to about 40% within the 68 h. From 98 h-168 h, the concentration reduced to below 20%. This population growth was higher than either 10 ppm or 20 ppm. This indicated that the increase in initial concentration has caused the mixed culture to increase the rate of degradation of Pyr in the culture medium.

But, from Figure 4 (D) with a 60 ppm of Pyr concentration, the biomass grew within 24 h to about 10µg/mL and stabilised between (24-98 h), and Pyr concentration started dropping. At duration of 116 h, the concentration of Pyr has dropped to

about 40% while the population of the mixed culture increases from 10µg/mL to 65µg/mL. Meanwhile, the population started to drop in the 168 h period and the remaining concentration of Pyr was about 20%, which is 12 ppm. Even though growth has occurred, reduction in concentrations of Pyr in this culture was confusing. Because, it is very difficult to verify a situation like this: except using the reduction in concentration of Pyr. Maybe, the Pyr was used within this period to synthesise some basic enzymes to enable the degradation of Pyr in the culture medium.

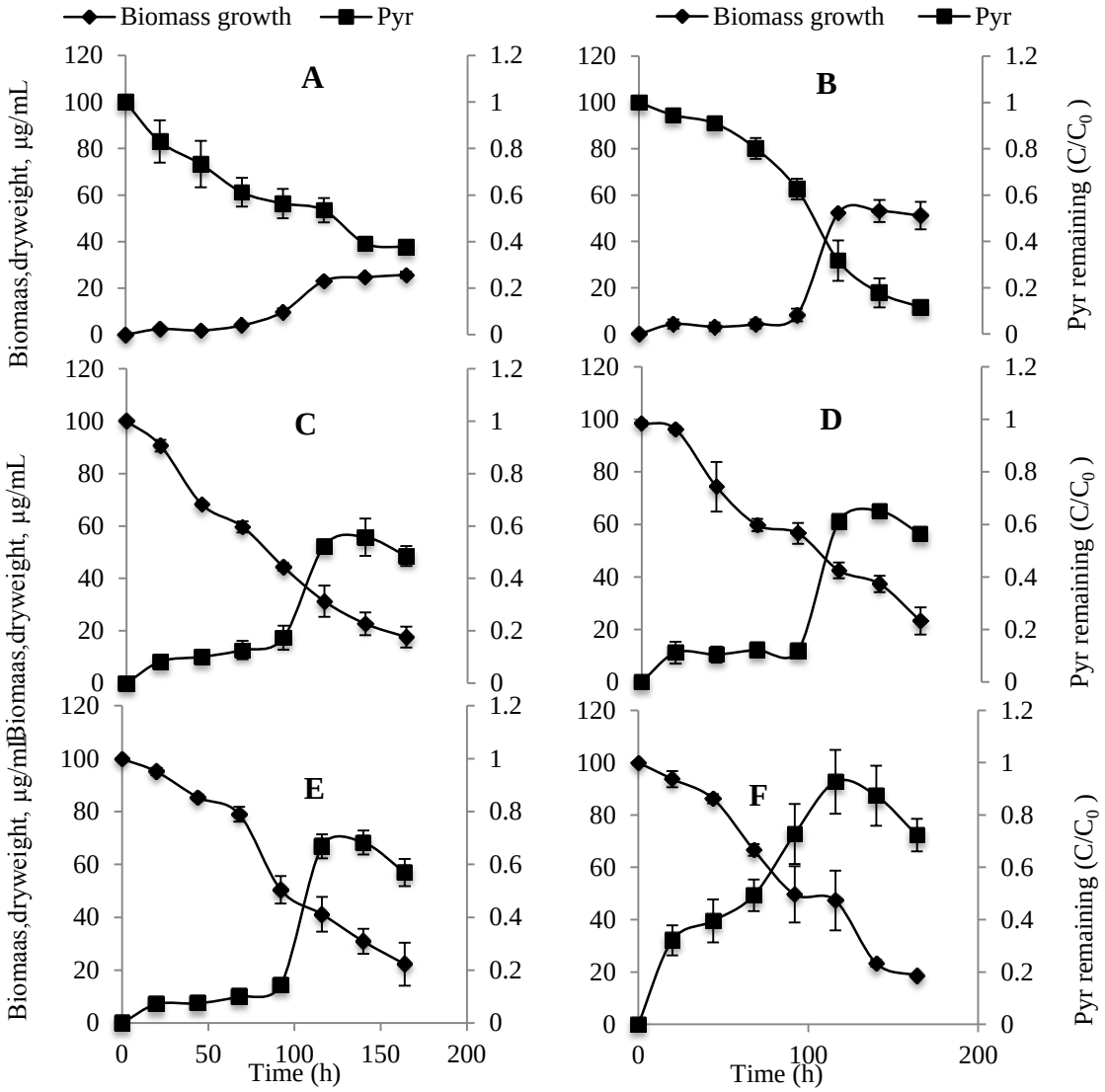

Figure 4: Change in biomass concentration and in Pyr concentration at: (A) 10 ppm; (B) 20 ppm; (C) 40 ppm; (D) 60 ppm; (E) 80 ppm; (F) 100 ppm: Each data point represents the mean standard error of three replicates

On the other hand, Figure 4 (E) with 80 ppm concentration of Pyr had a longer lag phase of 96 h and biomass increased slowly from 8µg/mL to 15µg/mL. The exponential phase started from 96 h and reached up to 120 h with biomass concentration of 70µg/mL and stabilised for 24 h, subsequently declined. The concentration of Pyr was dropped linearly from 90% to 20% at the end of 168 h.
With concentration of Pyr increased to 100 ppm, the degradation curve shows no

clear lag phase, as shown in Figure 4 (F). The mixed culture population increases within the 24 h period to about 38µg/mL and the Pyr concentration started to drop by 10% from the initial concentration. While the population of bacteria increases, the concentration of Pyr linearly decreases as the degradation continues. By the end of the experiment at 150 h, the concentration of Pyr has reduced to about 20% and the population of the mixed culture reached to about 100µg/mL.

3.2.1 Estimation of Kinetic Parameters

The estimation of kinetics parameters was done based on curve fitting using equation 2.8 and 2.9. The residual substrate concentration and biomass concentration data were used to estimate µ and k, which are the specific growth rate and degradation rate constant respectively. The values of the parameters are shown in Table 2.

Table 2: Estimated growth rate and first-order degradation constants

No	Pyr Concentration ppm	µ $\mu g(Lh)^{-1}$	r^2	k $mg(Lh)^{-1}$
1	10	0.0475	0.9439	0.0029
2	20	0.0487	0.7990	0.0073
3	40	0.0297	0.8602	0.0045
4	60	0.0358	0.7852	0.0054
5	80	0.0389	0.8384	0.0055
6	100	0.0180	0.9960	0.0055

It can be seen that the estimated µ is higher at substrate concentration of 20 ppm than at any other substrate concentration between 30-100 ppm but closer to µ at 10 ppm. Moreover, more than 90% of the data fitted well with respect to biomass growth at 10 ppm and 100 ppm, while substrate consumption or degradation rate constant, k was the lowest (0.0029 mg/Lh) at 10 ppm, as compared to other substrate concentrations. However, at 100 ppm substrate concentration, there was highest r^2 almost equals to 1, while its µ is lower than at other concentration below 100 ppm. On the average over 70% of the biomass growth data described the specific growth rate, µ. However, the specific growth rate, µ increased between substrate concentration of 10 ppm and 20 ppm, from 40 ppm to 80 ppm but decreased at a substrate concentration of 100 ppm. Moreover, specific growth rate is at average of 0.0364 µg/Lh and substrate consumption rate or degradation rate constant ranges from (0.0029 mg/Lh-0.0073 mg/Lh) and at average of 0.0052 mg/Lh. Therefore, a concentration higher than 100 ppm has negative effect on the biomass growth as well as rate of degradation of Pyr, which has been confirmed earlier.

Based on estimating the μ_{max} and K_s using the Lineweaver-Burk plot (Lineweaver and Burk 1934). This is a double reciprocal plot developed by Lineweaver and Burk (1934) as a useful method of graphical analysis to determine Michaelis-Menten kinetics parameters of K_m and V_{max} and Monod kinetics parameters of K_s and μ_{max}. From the plot in Figure 5 (A and B), the line of best fit for concentration range between

(100 ppm and 700 ppm) and (10 ppm and 100 ppm), with r^2, 0.9976 and 0.9976 respectively. From the Lineweaver-Burk plot, the kinetics parameters were calculated and value for μ_{max} and K_s are shown in Table 3.

Table 3: Kinetics Parameters

No	Concentration range, ppm	μ_{max} h^{-1}	K_s day^{-1}
1	10-100	1.6740	55.67
2	100-700	0.8579	79.75

The fitted Monod model and the experimental data are shown in Figure 6 respectively. This study found that Pyr concentration has a very significant effect on the biomass growth. Considering the hydrophobic nature of Pyr, there is very limited growth if the concentrations of Pyr were above 200 ppm.

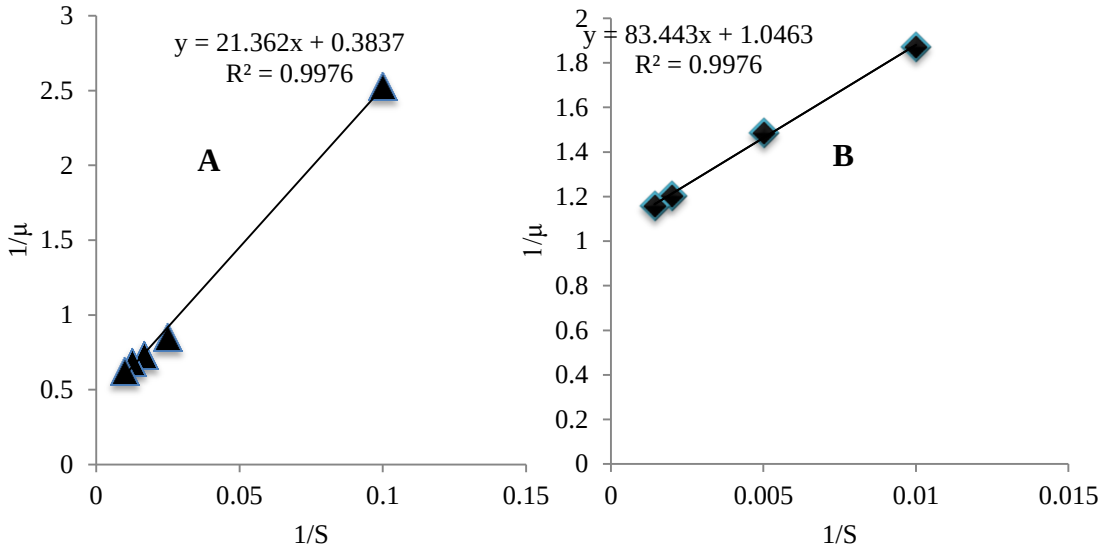

Figure 5: The Lineweaver-Burk plot for Pyr concentration between (A (100-700 ppm) and B (10-100 ppm)

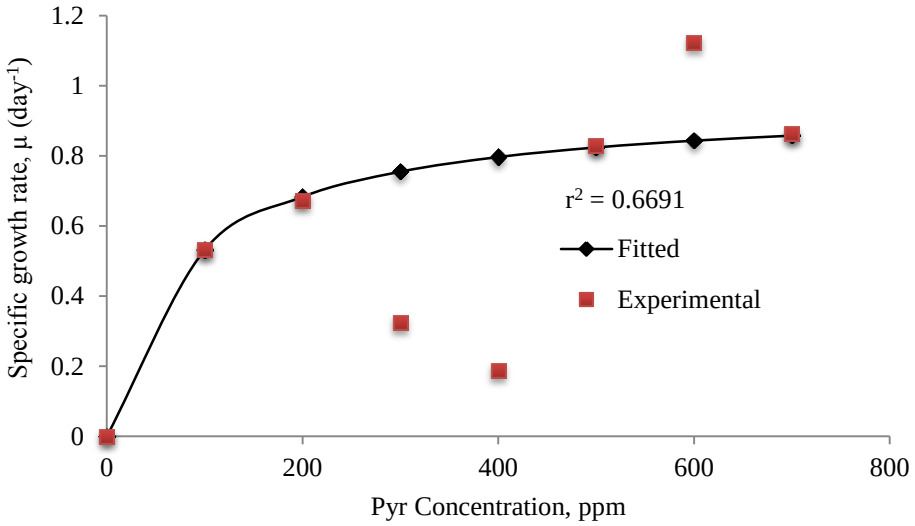

Figure 6: Growth rate of mixed culture vs Pyr concentration (100-700 ppm) and fitted Monod equation

Even though there was growth, the inconsistent nature of the biomass population and fluctuating pattern of the growth curve confirmed that high concentration of Pyr has significant effect on the growth of biomass, subsequently overall degradation of Pyr at higher concentration as found by this study. However, when the concentration was reduced from the higher range of (100-700 ppm) to a range lower between 10 to 100 ppm, the biomass growth has been very consistent and there is increase in biomass concentration as the concentration of Pyr increases. This indicates that within this range of concentration, the mixed culture growth was very similar to normal bacterial growth: with clear lag phase, log phase and stationary phase. The growth and degradation data were fitted to Monod model and about 94% of the data was described by the Monod equation in the case of the lower concentration range, and about 68% of the data was also described by the Monod equation with respect to higher concentration range. Attempt has been made to fit both the data on other models such as Haldane, Aiba, Yano, and Edwards did not fit very well, and over 80% of the data could not be described by those models. Modelling mixed culture of bacteria degrading organic compounds has been criticised, because of the black box nature of the degradation process (Dimitriou-Christidis 2005). No one can tell with certainty, who does what? Because, kinetics parameters are assumed to represent a total biomass constituting organisms with varying degree of enzymatic activities (Kovarova-Kovar & Egli 1998). This is the reason why many works conduct the kinetics study of only single and pure culture studies. But, in the real world it could be possible to have mixtures of bacterial attacking a single organic compound, since there is possibility of intermediate metabolites becoming a substrate for other organisms within the community.

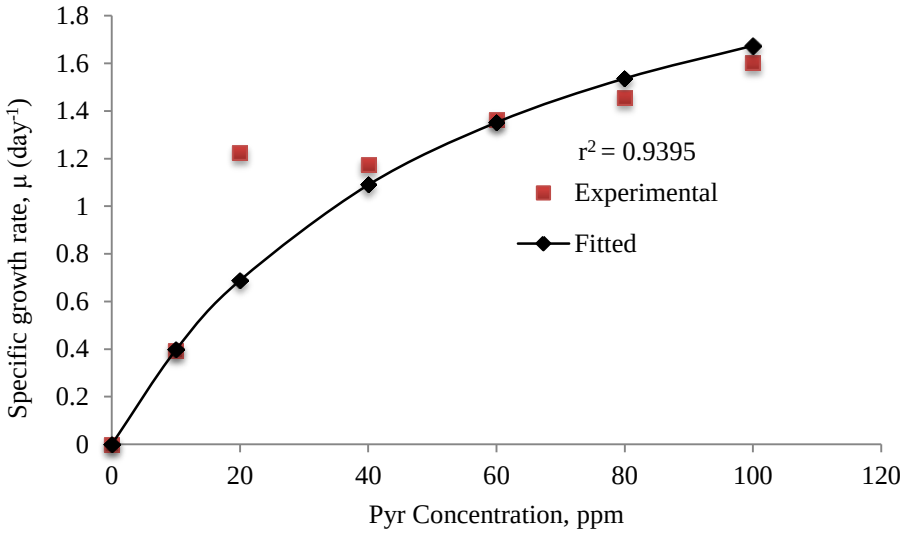

Figure 4.8: Growth rate of Mixed culture vs Pyr concentration (10-100 ppm) and fitted Monod equation

A biodegradation model using mixed culture of bacteria degrading PAH is very limited. Nevertheless, many works used Monod-type kinetics to describe kinetics of microbial degradation of PAHs (Dimitriou-Christidis *et al*. 2007, Mulder *et al*. 2001) Other models incorporate rate of dissipation or dis- solution of PAH due to their hydrophobicity using their individual octanol-water partition coefficient. Of course, it is very important to consider rate of dissolution, as there is possibility for some PAH to attach to cell or their bioavailability (Volkering *et al*. 1992). For example, Volkering *et al*. (1992) obtained values of C_{max} and growth rate for Nap, Phn and Ant. But for Ant, which is poorly soluble like Pyr, was modelled by (Wick *et al*. 2011) and obtained a very good prediction for substrate bioavailability and microbial growth. Although, this work did not carry out substrate bioavailability, considering Pyr more hydrophobic than some of the lower-molecular-weight (LWM) PAHs, the result could still represent the unique characteristics of mixed culture. Other aspect, which may be worth mentioning with regards to fitting mixed culture growth on a single organic compound using Monod growth kinetics is that, Monod kinetics, is just a very simplified representation of a very complex environment. Therefore, variability of kinetics parameters in describing a complex system especially when dealing with mixed culture may not be a surprise.

4. Conclusion

This study confirmed that biomass growth is affected by higher Pyr concentration (200-700 ppm). The growths were inconsistent and degradation was greatly affected by concentrations. However, with a concentration of Pyr between 10-100 ppm, a very convincing biomass growth and degradation of Pyr were obtained. First-order rate constant, k was higher (0.0487 mg/lh) with a substrate concentration of 20 ppm than other concentrations. The average degradation rate constant is 0.0029 mg/Lh for all the concentrations tested. This indicated that the mixed culture could degrade over

0.0696 ppm of Pyr per day. It also confirmed that kinetics of microbial degradation was partially fitted into Monod model. The data can be used to estimate biodegradation of Pyr by a mixed culture and preliminarily estimation of degradation rates.

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